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Increased activities of antioxidant enzymes and decreased ATP concentration in cultured myoblasts with the 3243A → G mutation in mitochondrial DNA

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Abstract

The MELAS syndrome (mitochondrial encephalomyopathy, lactic acidosis and stroke-like episodes) is most commonly caused by the 3243A → G mutation in mitochondrial DNA, resulting in impaired mitochondrial protein synthesis and decreased activities of the respiratory chain complexes. These defects may cause a reduced capacity for ATP synthesis and an increased rate of production of reactive oxygen species. Myoblasts cultured from controls and patients carrying the 3243A → G mutation were used to measure ATP, ADP, catalase and superoxide dismutase, which was also measured from blood samples. ATP and ADP concentrations were decreased in myoblasts with the 3243A → G mutation, but the ATP/ADP ratio remained constant, suggesting a decrease in the adenylate pool. The superoxide dismutase and catalase activities were higher than in control cells, and superoxide dismutase activity was slightly, but not significantly higher in the blood of patients with the mutation than in controls. We conclude that impairment of mitochondrial ATP production in myoblasts carrying the 3243A → G mutation results in adenylate catabolism, causing a decrease in the total adenylate pool. The increase in superoxide dismutase and catalase activities could be an adaptive response to increased production of reactive oxygen species due to dysfunction of the mitochondrial respiratory chain.

Keywords: Mitochondrial disease; MELAS syndrome; Muscle; ATP production; Superoxide dismutase; Catalase

1. Introduction

The classical clinical phenotype of MELAS syndrome includes encephalomyopathy, lactic acidosis and stroke-like episodes [1], but multiorgan involvement and phenotypic variability are also characteristic of this disease [2]. The 3243A → G mutation in mitochondrial DNA, which is the most common cause of the MELAS syndrome [3], has been previously shown to result in impairment of transcription termination [4] and protein synthesis [5], and the most common biochemical consequence of the muta-

Abbreviations: MELAS, mitochondrial encephalomyopathy, lactic acidosis and stroke-like episodes; MERRF, myoclonic epilepsy with ragged red fibres; PTP, mitochondrial permeability transition pore; ROS, reactive oxygen species; SOD, superoxide dismutase

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tion is a decrease in the activities of respiratory chain complexes I and IV [6–8]. Decreased production of ATP has been suggested as a major pathophysiological phenomenon in mitochondrial diseases and in the MELAS syndrome [9], this being supported by the demonstration of mitochondrial myopathy in adenine nucleotide translocator (Ant1, heart/muscle isoform) knock-out mice [10]. No decrease has been observed in ATP production in cultured fibroblasts with the 3243A→G mutation [11,12], but the situation may be different in cells derived from tissues that have a higher energy demand and are affected in mitochondrial myopathies.

The production of oxygen-derived free radicals may have a significant role in the pathophysiology of neurodegenerative diseases [13,14] and mitochondrial diseases. Partial reduction of oxygen results in the formation of reactive oxygen species (ROS), including superoxide ($O_2^{\bullet -}$), peroxide (O_2^{2-}) and hydroxyl radicals ($OH^{\bullet}$). There are several possible sites of cellular superoxide formation, including complex I [15] and complex III [16] of the mitochondrial respiratory chain. Mammalian cells have several defence mechanisms for use against attacks by these toxic oxygen radicals. The mitochondrial superoxide dismutase (manganese SOD) and cytosolic superoxide dismutase (copper/zinc SOD) catalyse the conversion of superoxide to hydrogen peroxide, which is degraded by catalase in peroxisomes and glutathione peroxidase in the cytosol and mitochondria. Cells carrying mtDNA mutations have been observed to be more sensitive to oxygen stress than control cells [17]. Furthermore, a higher rate of peroxide production and an increased rate of apoptosis have been reported in the presence of high oxygen concentrations in cybrids carrying the 3243A→G mutation [18].

The purpose of this study was to examine the pathophysiological consequences of the oxidative phosphorylation defect caused by the 3243A→G mutation by determining the concentrations of high energy phosphates and the activities of ROS scavenging enzymes in cultured myoblasts. ATP and ADP were measured to obtain an estimate of the cellular energy state and the adenylate pool in myoblasts. The superoxide dismutase and catalase activities were also measured, as it has been suggested that an increase in the activity of superoxide dismutase may indicate increased free radical formation [19].

2. Materials and methods

2.1. Muscle cell culture

The study was approved by the Ethical Committee of the Medical Faculty of the University of Oulu and informed consent was obtained from the patients. Myoblast cell cultures (Me1, Me4, Me5 and Me8) were established from four patients (P1, P4, P5 and P8, respectively) with the 3243A→G mutation in their mtDNA and from four controls (Co1–Co4). Eligible controls were under 50 years of age and without any chronic diseases. The muscle samples obtained from the patients and controls were first dissected free of any visible connective tissue and cut into 1 mm diameter pieces, which were plated and grown for 2–3 weeks in Earle's minimum essential medium (Sigma, St. Louis, MO, USA) containing 15% foetal bovine serum, 4 mM L-glutamine, 1 mM pyruvate, amphotericin B (0.25 μg/ml), streptomycin (100 μg/ml) and penicillin (100 U/ml). The cells were detached with trypsin-EDTA and preplated for 10 min in order to remove possible contaminating fibroblasts. The medium was then transferred to new culture dishes (Nunclon, NalceNunc, Roskilde, Denmark). Confluent cells were washed with phosphate-buffered saline (PBS), detached with trypsin-EDTA (Sigma), divided and inoculated into two new dishes. The cell cultures were used in passages three to seven in all experiments.

2.2. Blood samples

Blood samples were obtained from 17 patients with the 3243A→G mutation (age 45±15 years, mean±S.D.) and from 13 controls (age 50±11 years, mean±S.D.). Haemoglobin concentration and SOD activity were measured immediately.

2.3. High energy phosphates

$HClO_4$ was added to the cell cultures for the metabolite assays. The cells were scraped with a rubber policeman and the cell suspension was sonicated and

neutralised with K_2CO_3-triethanolamine. The sonicated cells were centrifuged for 10 min at 3200×*g* and the protein concentration in the pellet was measured. ATP was measured spectrophotometrically by detecting NADPH formation in the reactions catalysed by hexokinase and glucose 6-phosphate dehydrogenase [20]. ADP was measured by pyruvate kinase and lactate dehydrogenase and the decrease in absorbance of NADH was measured spectrophotometrically at 340 nm minus 385 nm with an Aminco DW-2 dual wavelength spectrophotometer [21].

2.4. Determination of mutant heteroplasmy

The cells were scraped from the culture dishes with a rubber policeman and total DNA was extracted with the QIAamp Blood Kit (Qiagen, CA, USA). The degree of 3243A→G mutant heteroplasmy was measured in samples from three or four passages of each cell line cultured in parallel to those used in experiments [22]. The degrees of the mutant heteroplasmy in the muscle samples from the patients were also determined.

2.5. Superoxide dismutase and catalase activities

Cells were scraped from culture dishes with a rubber policeman, dissolved in potassium phosphate buffer (pH 7.0) and sonicated on ice. Soluble proteins were quantified spectrophotometrically by a dye binding assay (Bio-Rad protein assay, Bio-Rad Laboratories, Hercules, CA, USA).

Total superoxide dismutase activity was measured from myoblast cell lysate and from blood cells by a method which employs xanthine and xanthine oxidase to generate superoxide radicals, which react with 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazolium chloride to form a red formazan dye measurable spectrophotometrically at 505 nm (Ransod kit, Randox Ltd., Antrim, UK) [23]. Superoxide dismutase activity was measured in terms of the inhibition of formazan formation. The inhibition levels were converted to SOD units using a standard curve, prepared with SOD standards provided by the manufacturer.

Catalase activity was measured from sonicated myoblast lysate. Degradation of hydrogen peroxide was detected spectrophotometrically at 240 nm as previously described [24]. Catalase activity was expressed as a first-order constant per unit of soluble protein (mg^{-1} s^{-1} ml).

The statistical significances of differences were calculated using Student's two-tailed *t*-test.

3. Results

3.1. Heteroplasmy

The clinical phenotypes of the patients with the 3243A→G mutation and the degrees of mutant heteroplasmy are shown in Table 1. It should be noted that although the muscle sample from patient P4 had a mutant heteroplasmy of 62%, the 3243A→G mutation had disappeared in two out of the four myoblast culture dishes that were examined. Disappearance of the mutant genome was not observed in any other cell lines, but the degree of mutant heteroplasmy was lower in cultured myoblasts than in the corresponding muscle samples. A similar decline of mutant heteroplasmy has been previously observed

Table 1
Clinical features and myoblast cell lines of the patients with the 3243A→G mutation

Patient	Cell line	Sex	Age	Clinical phenotype[a]	Degree of 3243A→G heteroplasmy (%)	
					in muscle	in myoblasts
P1	Me1	F	50	Healthy	50	31 ± 9
P4	Me4	M	64	C D G H L M S	62	21 ± 19[b]
P5	Me5	F	40	B C E G L M S	80	41 ± 14
P8	Me8	F	55	C G	67	40 ± 19

[a]Abbreviations: B, basal ganglia calcifications; C, cognitive decline; D, diabetes mellitus; E, epilepsy; G, short stature; H, cardiac hypertrophy; L, lactic acidosis; M, clinical myopathy; S, sensorineural hearing impairment.
[b]Two out of four cell lines had lost their mutant heteroplasmy. Data are means ± S.D. of three determinations.

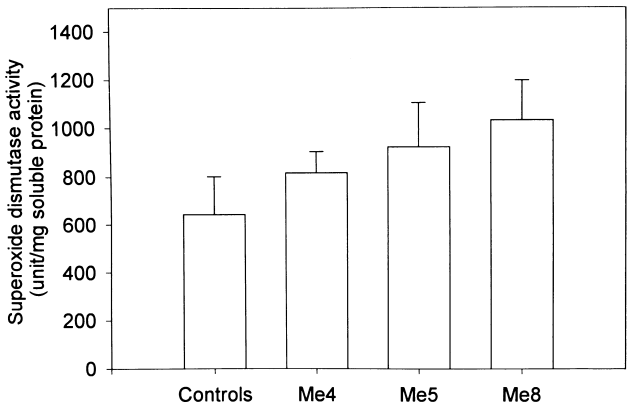

Fig. 1. Superoxide dismutase activities in cultured myoblasts. A significant difference compared with the controls is observed in all cell lines with 3243A→G.

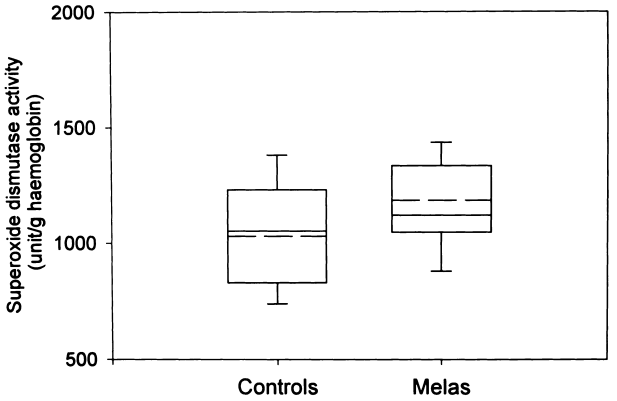

Fig. 2. Superoxide dismutase activity in blood cells of patients with 3243A→G and controls. The solid lines of the boxes show the 25%, 50% and 75% percentiles and dotted line represents the mean. The whiskers show the 5% and 95% percentiles.

in cultured myoblasts with the 3243A→G mutation [25].

3.2. Concentrations of high energy phosphates in myoblasts

The ATP concentration (Table 2) was significantly lower in Me4 ($P < 0.002$) and Me5 ($P < 0.001$) than in two of the control cell lines, but was higher in the Me1 cell line ($P < 0.02$) than in the controls. This is explained by the low degree of mutant heteroplasmy in the Me1 cells. Patient P1 was also clinically healthy.

The total ADP concentration (Table 2) was significantly lower in Me4 ($P < 0.02$) than in the two relevant control cell lines, but not in Me1 or Me5. The ATP/ADP ratios (Table 2) did not differ significantly between the control and MELAS myoblasts. The total ATP+ADP pool (Table 2) was significantly lower in Me4 ($P < 0.001$) and Me5 ($P < 0.001$) than in the control cells, but higher in Me1 ($P < 0.03$).

3.3. Activities of antioxidant enzymes

Total superoxide dismutase activity was 644 ± 130 U/mg soluble protein in the control myoblasts, compared with 819 ± 87, 924 ± 181 and 1033 ± 165 U/mg soluble protein in Me4, Me5 and Me8, respectively (Fig. 1). It was elevated in all the mutant cell lines (Me4 $P < 0.02$, $n = 7$; Me5 $P < 0.002$, $n = 7$ and Me8 $P < 0.001$, $n = 7$) relative to the two control cell lines, $n = 14$. Superoxide dismutase activity tended to be slightly higher in the blood of patients with 3243A→G (Fig. 2) than that in the controls, 1186 ± 253 vs 1030 ± 242 U/mg soluble protein, respectively, although the result did not reach statistical significance ($P < 0.1$).

Catalase activities in cultured myoblasts with 3243A→G were 3.4 ± 0.4, 10.1 ± 2.1 and 9.5 ± 2.6 U/mg soluble protein in Me4, Me5 and Me8, respectively (Fig. 3), while the activity in the control cells was 4.0 ± 1.4 U/mg soluble protein. Catalase activity

Table 2
Concentrations of high energy phosphates in cultured myoblasts

Cell line	ATP concentration (nmol/mg soluble protein)	ADP concentration (nmol/mg soluble protein)	ATP/ADP ratio	ATP+ADP concentration (nmol/mg soluble protein)
Controls	45.0 ± 10.0, $n = 14$	13.9 ± 8.1, $n = 11$	3.8 ± 1.8, $n = 11$	60.2 ± 11.0, $n = 11$
Me1	62.7 ± 20.5	18.7 ± 8.0	3.6 ± 1.3	81.4 ± 27.1
Me4	28.5 ± 10.7	5.7 ± 3.0	4.4 ± 1.4	34.2 ± 12.1
Me5	19.5 ± 4.6	9.5 ± 6.1	2.9 ± 2.1	29.0 ± 8.7

Data are means ± S.D. n is the number of cell culture dishes examined. For all cell lines with the 3243A→G mutation, $n = 7$.

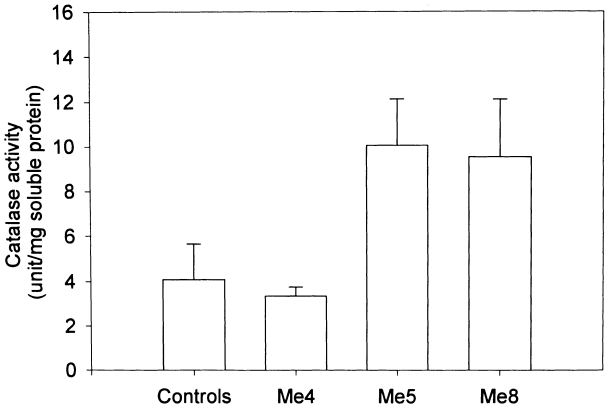

Fig. 3. Catalase activities in cultured myoblasts. Catalase activity was increased in myoblasts with a high degree of 3243A→G mutant heteroplasmy (Me5 and Me8), but not in cell line Me4, with lower mutant heteroplasmy.

was significantly higher in cell lines Me5 ($P < 0.001$, $n = 7$) and Me8 ($P < 0.002$, $n = 7$) than in the two control cell lines ($n = 14$). The cell line Me4 did not differ from the controls in this enzyme activity, however.

4. Discussion

Our results confirmed that the 3243A→G mutation in mitochondrial DNA leads to impaired ATP production in myoblasts. The impairment was more severe in cell lines with a higher degree of mutant heteroplasmy, whereas the ATP concentration was not lowered in the cell line Me1 relative to the controls. The normal biochemical phenotype in this cell line suggests that the respiratory chain defect is not severe enough to exceed the threshold at which decreased ATP production occurs and the MELAS phenotype is expressed.

No difference in ATP production has been found between cultured MELAS and control fibroblasts [11,12], although decreased ATP concentrations have been observed in fibroblasts with the 3243A→G mutation after treatment with gramicidin [11], a potassium ionophore which increases ATP consumption. Gramicidin uncouples the mitochondria and increases the workload of the plasma membrane Na^+/K^+-ATPase. On the other hand, we found a difference in ATP production in nascent cultured myoblasts that is most likely explained by the high energy demand of muscle cells. Myoblasts are an appropriate cell line to be used in metabolic studies of mitochondrial diseases, because resting energy consumption is high and muscle cells are easily manipulated in cultures.

It is significant that the proportional changes in cellular total ATP and ADP concentrations were similar, so that the ATP/ADP ratio did not change. If the ATP decrease were solely due to cellular deenergisation, a corresponding ADP increase should be observed. This was not the case, however, and furthermore, the ADP concentration was significantly lower in the Me4 cell line than in the controls. The free ADP concentration in muscle cells is very low and most of it is bound to contractile proteins [26], so that changes in the free ATP/ADP ratio have only a small effect on total ADP concentration. The decrease in ADP with a concomitant decrease in ATP can then only be accounted for by a loss of adenine compounds from the cell. There is previous evidence that complex I inhibition results in a rapid decrease in the overall size of the adenine nucleotide pool in nerve cell cultures [27], similar observations have been documented in respiration-deficient hamster fibroblasts [28]. It has been proposed that the activities of enzymes degrading AMP, adenylate deaminase and AMP nucleosidase, increase in conditions where the adenylate energy charge is decreased [29,30]. Furthermore, it is known that purine nucleotide degradation is initiated under conditions of low ATP and high AMP concentrations [31,32].

Superoxide dismutase and catalase activities were significantly higher in cell lines Me5 and Me8, whereas only superoxide dismutase increased in cell line Me4, in which the mutant heteroplasmy was lower. Inhibition of complexes I [15] and III [16] contributes to mitochondrial ROS formation, while decreased activities of complexes I and IV in the respiratory chain [6–8] are the most common biochemical defects associated with the 3243A→G mutation. This would be in accord with increased ROS formation in cultured myoblasts with 3243A→G and fits with the notion that catalase and superoxide dismutase (MnSOD) [19,33] are regulated in an adaptive way, i.e. increased ROS formation leads to increased synthesis of catalase and superoxide dismutase. The fact that superoxide dismutase activity tended to be slightly higher in the blood cells of patients with

the 3243A→G mutation can be explained by the generally lower degree of mutant heteroplasmy in blood cells than in muscle cells [3].

Our results suggest that an increased amount of oxygen free radicals is one of the major biochemical disturbances in the MELAS syndrome. Cybrids with 3243A→G have been shown to be more sensitive to hydrogen peroxide-induced damage [17], and the production of H_2O_2 increases in cybrids with the same mutation at a high oxygen concentration [18]. Furthermore, we found a decrease in high energy phosphates, and previous studies have demonstrated decreased mitochondrial membrane potential [34,35]. Also disturbances in calcium concentration [36] have been observed in cells with 3243A→G. Decreased mitochondrial membrane potential, increased mitochondrial Ca^{2+} concentration and increased production of ROS is known to increase the opening of the mitochondrial permeability transition pore (PTP) [37]. In a reconstituted membrane system, a high ADP concentration inhibited the adenine nucleotide translocator to form a PTP [38]. The observations made on 3243A→G cells [34,35], the properties of the adenine nucleotide translocator [38] and the results obtained here suggest that the cellular homeostasis is disturbed in such a way that the probability of PTP opening may be increased. It needs to be evaluated whether this affects the adenylate compartmentation and their total pool size.

The pathogenic cascade evolving from the 3243A→G mutation leads to impairment of transcription termination [4], a decrease in protein synthesis [5] and decreased activities of the respiratory chain complexes I and IV [6–8]. As shown here, these defects lead to impaired ATP production, a decrease in the total adenylate pool and increased formation of reactive oxygen species. These pathogenic events may partly determine the biochemical phenotype of cells and the clinical phenotype of the patients, but, in addition, they may trigger other cellular responses, the roles of which in the pathogenesis of mitochondrial diseases remain unknown.

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